Specification

Novel Protoporphyrinogen Oxidase Tolerant To Photobleaching Herbicide

Field Of The Invention

The present invention relates to a novel protoporphyrinogen oxidase tolerant to light-requiring herbicide, a gene which codes the protein, a recombinant vector comprising the gene and a transformant by the vector.

Background Of The Invention

Protoporphyrinogen oxidase is an enzyme which convert protoporphyrinogen IX into protoporphyrin IX, in a tetrapyrrole synthesis system which exists universally in all organism.

In this synthesis system, heme is synthesized in case of a microorganism and an animal, on the other hand, chlorophyll in addition to heme is synthesized in case of a plant. This enzyme is thought to be a target enzyme of photobleaching herbicide, and the identification of the enzyme and the isolation of the gene has not been performed until now. *HemG* of *E.coli* has been isolated as a protoporphyrinogen oxidase gene in 1993 (Sasarman, A. *et al* (1993) *Can. J. Microbiol.* 39:1156-1161.), and *HemY* of *B.subtilis* has been isolated as a protoporphyrinogen oxidase gene in 1994 (Dailey, T. A. *et al.* (1994) *J. Biol. Chem.* 269:813-815.). In eukaryote, cloning of human protoporphyrinogen oxidase gene has been conducted in 1995 (Nishimura, K. *et al.* (1995) J. Biol. Chem.270:8076-8080.), and in the same year, cloning of mouse protoporphyrinogen oxidase gene has been conducted (Taketani, S. *et al.* (1995) *Eur. J. Biochem.* 230:760-765.). In plant, cloning of *Arabidopsis thaliana* and corn protoporphyrinogen oxidase gene has been conducted in 1995 (WO 96/34659).

Further, a gene of *Arabidopsis thaliana*... Heynh. and corn protoporphyrinogen oxidase tolerant to photobleaching herbicide has already been obtained by screening system using *E. coli*.

The screening of a gene of *Arabidopsis thaliana* and corn protoporphyrinogen oxidase tolerant to photobleaching herbicide (WO 95/34659) has been done by using *E. coli*. Thus, it is not clear whether sufficient activity of such gene can be performed in a plant cell, and whether the tolerance is sufficient.

Disclosure Of The Invention

When a plant tolerant to photobleaching herbicide is produced by using a recombinant DNA technique, the protoporphyrinogen oxidase gene tolerant to photobleaching herbicide, derived from a higher plant can be a strong candidate. Protoporphyrinogen oxidase derived from the other organism is largely different, for example, the protoporphyrinogen oxidase derived from *E. coli* is different in that the molecular weight is apparently smaller compared with the enzyme from other organism. The protoporphyrinogen oxidase derived from *B. subtilis* is soluble and very different from a membrane-bound type protoporphyrinogen oxidase from the other organism in that the former is soluble.

Further, the protoporphyrinogen oxidase derived from an animal has special nature that it is transported into mitochondria, in spite that it has no transit peptide.

In producing a plant tolerant to photobleaching herbicide by using recombinant DNA technique, using of the protoporphyrinogen oxidase which is derived from a higher plant is preferable, in view of such differences of the nature in the protoporphyrinogen oxidase derived from each organism.

On the other hand, it is not clear whether the resistant gene (WO 97/04088) derived from *Chlamydomonas*, an unicellular organism, is protoporphyrinogen oxidase, and whether the gene can give a tolerance to a higher plant.

Therefore, preferable gene is thought to be such a gene obtained from a process in which a plant cell or a plant is screened with herbicides, the resistant type gene in the plant cell or plant body is confirmed to have a sufficient biological activity and a high tolerance to photobleaching herbicide in a plant cell, then, the gene is isolated by using a certain method.

Accordingly, the problem of the present invention is to provide a protoporphyrinogen oxidase highly tolerant to photobleaching herbicides, a gene encoding the enzyme, a recombinant vector comprising the gene and a transformant by the vector.

The present inventors have extensively studied about a higher plant protoporphyrinogen oxidase to solve the above-described problem. As the result, the present inventors have taken a steps in which a plant cell of plant body is screened under the existence of herbicides, the resistant type gene in the plant cell or plant body is confirmed to have a sufficient biological activity and a high tolerancey to photobleaching herbicide in a plant cell, then, the gene is isolated by using a certain method. In more detail, the present inventors have extensively studied about protoporphyrinogen from a higher

plant, especially tobacco to solve the above-described problem. As the result, the present inventors have succeeded in cloning of protoporphyrinogen oxidase tolerant to photobleaching herbicide from tobacco. The gene of the present invention is novel, and when tolerance of the gene product to photobleaching herbicide is compared with the tolerance of the known protoporphyrinogen oxidase derived from *Arabidopsis thaliana*, it has now been found that the tolerance of the present gene product is very superior, in spite of large structural similarity of both genes. Accordingly, the above-described problems can be solved by using the gene of the present invention.

The present invention provides protoporphyrinogen oxidase, derivatives thereof or mutants thereof, comprising the amino acid sequence represented by SEQ ID No.2 or mutated peptide derived therefrom by deletion, addition, substitution, *etc.* of one or more amino acids in the above amino acid sequence and having an enzyme activity substantially equivalent to that of the protoporphyrinogen oxidase tolerant to photobleaching herbicide.

The protoporphyrinogen oxidase of the present invention comprising the amino acid sequence represented by SEQ ID No.2 includes mutated peptide in which deletion, addition, substitution, *etc.* of one or more amino acids in the above amino acid sequence is observed, that is, mutated peptide discovered in the nature or artificially modified mutated peptide, provided that the mutated peptide is not damaged in substantially same enzyme activity and tolerance to photobleaching herbicide.

The present polypeptide is thought to be an enzyme existing in a chloroplast. In general, almost all protein existing in intracellular organelle, such as a chloroplast, is encoded in a genome of nuclear, and it is translated in cytoplasm, then, by the function of a transportation signal, called transit peptide, existing in N-terminal, it is transported to these intracellular organelle. After the transportation, the transit peptide is cleaved to become a mature protein. Accordingly, it can be thought that transit peptide exists in N-terminal of the polypeptide of the present invention. Further, essential part by which biological activity is expressed is a part in which transit peptide is excluded, i.e., a mature protein, thus, the transit peptide does not relate to the activity. Accordingly, the present invention comprises a mature protein in which transit peptide is deleted.

In more detail, the present invention provides the protoporphyrinogen oxidase gene comprising the nucleotide sequence represented by SEQ ID No.3 or the nucleotide sequence in which one or more nucleotide in the above nucleotide

sequence is deleted, added, or substituted and which encode a polypeptide having an activity substantially equivalent to that of the protoporphyrinogen oxidase.

Further, the DNA sequence encoding the above polypeptide of the present invention includes cDNA encoding objective protoporphyrinogen oxidase, chromosomal DNA comprising intron and exon, DNA in which exon is connected with exclusion of intron, and further a synthetic DNA which is obtained by connecting oligonucleotide prepared artificially using a synthetic DNA method. When a synthetic DNA is prepared by using the synthetic DNA method, the objective DNA can be prepared by degeneration of genetic code, and by changing the nucleotide sequence of the gene, without changing encoding amino acid. Thus, the DNA of the present invention comprises all nucleotide sequences which is based on the degeneration of the gene encoding the objective polypeptide.

Further, present invention provides a recombinant vector comprising the above-described gene, a transformant by the above-described vector.

Brief Explanation Of Drawings

Figure 1 is schematic figure of pBNtPX-1

Figure 2 is schematic figure of pCR-HC and pCR-RC
▼:mutation in pCR-RC
c is mutated to T at 717th nucleotide in SEQ ID No.1, and alanine is mutated to valine at 231th amino acid)

Figure 3 is schematic figure of pBAtPX-C and pBAt-RC
▼:mutation in pBAtPX-RC
(same mutation as pAraC-1Val in WO 95/34659)

Figure 4 is schematic figure of pBI-NtPX-HC and pBI-NtPX-RC
▼:mutation in pBI-NtPX-RC
c is mutated to T at 717th nucleotide in SEQ ID No.1, and alanine is mutated to valine at 231th amino acid)

Figure 5 is schematic figure of pBI-AtPX-HC and pBI-AtPX-RC
▼:mutation in pBI-AtPX-RC
(same mutation as pAraC-1Val in WO 95/34659)

Best Mode For Utilization of The Invention

Hereinafter, the invention is described in more detail.

The general procedures for plant cell and tissue culture, or series of biological procedures, such as purification of mRNA, preparation of cDNA, cDNA library, recombinant DNA, determination of nucleotide sequence of DNA, or plant biological procedures, such as transformation of plant can be performed according to known literatures, for example, Plant Cell and Tissue Culture, Vasil, I. K. and Thorpe, T. A. Kluwer

Academic Publishers, 1994 *etc.*, or Molecular Cloning 2nd Edition, CSH Laboratory Press, Sambrook, J. *et al.*, 1989, Current Protocols in Molecular Biology, John Wiley & Sons, Ausubel, M. *et al. etc.*, or Plant Molecular Biology Manual, Gelvin, S.A. *et al.* Kluwer Academic Publishers, 1991, 1995 (Second edition) *etc.*

1. Photobleaching herbicide

The photobleaching herbicide used in the present invention is a herbicide which requires light for expressing herbicidal activity. When the photobleaching herbicide is applied to a plant, at first, the membrane in a cell is destroyed peroxidatively, then terrestrial part becomes white, and withered at last. In a cell treated by photobleaching herbicide, protoporphyrin IX was found to be accumulated (Matringe, M. And Scalla, R. (1988) *Plant Physiol.* 86:619-622.), and it became clear by the study thereafter that the target enzyme of the photobleaching herbicide is protoporphyrinogen oxidase which convert protoporphyrinogen IX to protoporphin IX in tetrapyrrole synthesis system (Matringe, M. *et al.* (1989) *Biochem, J.* 260:231-235.). At present, mode of action of the photobleaching herbicide is thought to be as follows. When the protoporphyrinogen oxidase in a plant cell is inhibited by a photobleaching herbicide, at first protoporphyrinogen IX, a substrate, is accumulated, then, protoporphyrin IX is produced in large amount from this protoporphyrinogen IX, non-enzymatically or by non-specific oxidation enzyme in the cytoplasm, which is non-sensitive to the photobleaching herbicide. This protoporphyrin IX introduces a photosensitization reaction under light, a large amount of active oxygen is generated, and it destroys a membrane peroxidatively to cause a plant wither (Scalla, R. and Matringe, Mm. (1994) *Rev. Weed Sci.* 6:103-132). From these mode of action, photobleaching herbicide is also called as a protoporphyrinogen oxidase (Protox) inhibitor or a porphyrin-generating type herbicide.

Compound having wide variety of structures belong to the photobleaching herbicides. As the representative compound, diphenyl ether series, oxadiazole series, pyridine series, pyrimidine series, cyclic imide series, triazole series, pyrazole series (Scalla, R. and Matringe, M. (1994) *Rev. Weed Sci.* 6:103-132) are included.

As the photobleaching herbicide of the present invention, triazole series and pyrazole series are preferable, and the pyrazole series compounds described below are most preferable.

Compound name

English name: ethyl 2-chloro-5-(4-chloro-5-difluoromethoxy-1-methyl-1H-pyrazol-3-yl)-4-fluorophenoxyacetate
(hereinafter referred to as Compound A. Described in Toku Kai Hei 3-163063)
English name: ethyl 2-[5-(4-chloro-5-difluoromethoxy-1-methyl-1H-pyrazol-3-yl)-2,4-dichloro-phenylamino]propionate
(hereinafter referred to as Compound B. Described in Toku Kai Hei 3-163063)
English name: 4-chloro-3-(4-chloro-2-fluoro-5-methoxyphenyl)-5-difluoromethoxy-1-methyl-1H-pyrazole
(hereinafter referred to as Compound C. Described in Toku Kai Hei 4-225937)
English name: 4-chloro-3-[4-chloro-2-fluoro-5-(2-propynyl)oxyphenyl]-5-difluoromethoxy-1-methyl-1H-pyrazole
(hereinafter referred to as Compound D. Described in Toku Kai Hei 3-163063)
English name: ethyl 2-[2-chloro-5-(4-chloro-5-difluoromethoxy-1-methyl-1H-pyrazol-3-yl)-4-fluorophenoxy]propionate
(hereinafter referred to as Compound E. Described in Toku Kai Hei 3-163063)
English name: 1-methylethyl 5-[4-bromo-1-methyl-5-(trifluoromethyl)-1H-pyrazol-3-yl]-2-chloro-4-fluorobenzoate
(hereinafter referred to as Compound F. Described in Toku Hyo Hei 10-502926)
English name: 4-chloro-3-(4-chloro-2-fluorophenyl)-5-difluoromethoxy-1-methyl-1H-pyrazole
(hereinafter referred to as Compound G. Described in Toku Kai Hei 3-72460)

2. Screening of tobacco callus tolerant to photobleaching herbicide

A plant can be differentiated from a protoplast, a cell, to a complete plant body, through callus which is a mass of cells. Such nature that a plant has is called as totipotency.

Further, as a characteristic of a plant cell in culture, it can be pointed out that the cell is very mutative, and such mutation is called as somaclonal variation. Accordingly, if such culturing cell is screened by a certain compound, for example, a herbicide, a herbicide-tolerant cell can be obtained. Further, the obtained herbicide-tolerant cell can be regenerated to a plant body by utilizing the totipotency. Many examples, such as, a glyphosate tolerant plant (Singer, S. R. And Mcdaniel, C. N. (1985) *Plant Physiol.* 78:411-416), a sulfonylurea tolerant plant (Chaleff, R. S. And Ray, T. B. (1984) *Science* 223:1148-1151), *etc.* are known. However, there are many cases that a culturing cell loses the totipotency by some reason during the culture. Further, it is difficult to introduce the herbicide tolerance into a plant of different species by using such methods. Thus, if a gene reponsible to

the tolerance to herbicide can be isolated from a cell tolerant to herbicide, the obtained gene can be introduced into several useful crops by known method, such as, the *Agrobacterium* method. As such a herbicide-tolerant gene, the following genes are known: a gene encoding an enzyme which detoxify the herbicide itself, for example, bromoxynyl detoxifying enzyme gene (Stalker, D. M. *et al.* (1988) *Science* 242:419-423.), bialaphos detoxifying enzyme gene (De Block, M. *et al.* (1987) *EMBO J.* 6:2513-2518); and a gene encoding an enzyme which detoxify some toxic substance produced by a herbicide, for example, superoxide dismutase gene (Furusawa, I. *et al.* (1987) *Plant Cell Physiol.* 25:1247-1252.); a herbicide tolerant type target enzyme gene, for example, sulfonylurea tolerant type acetolactate synthase (ALS) gene (Lee, K. Y. *et al.* (1988) *EMBO, J.* 7:1241-1248.), glyphosate tolerant type 5-enol pyruvylshikimate-3-phosphate synthase (EPSPS) gene (Comai, L. *et al.* (1985) *Nature* 317:741-744) *etc.*

3. Mode of action of herbicide tolerance in culturing Cell

As a mode of action of tolerance in culturing cell tolerant to a herbicide, acquisition of detoxification mechanism, change of drug permeability into a cell, over production of target enzyme and mutation of the target enzyme into herbicide tolerant type, *etc.* can be thought. On what mechanism the tolerance is based can be assumed by reviewing the presence of the accumulation of a precursor in the reaction which herbicides inhibit; by comparing tolerance against herbicides having various structures which show the same of different mode of action; by extracting a target enzyme from both tolerant cell and sensitive cell and comparing the enzyme activity; and by reviewing the sensitivity of the target enzyme *in vitro* against the herbicide used in the screening. As a result, if it have become clear that an useful herbicide tolerant gene exists, it becomes a useful gene source for isolating a herbicide tolerant gene.

4. Cloning method of the gene

As a method for cloning a gene, there is a method in which protein information is utilized, a method in which nucleic acid information is utilized and a genetical method. As the method in which protein information is utilized, there is a method in which a target protein is purified, and the expression library is screened out by using an antibody thereof, a method in which the amino acid sequence of the target protein is identified even in partial, and from the information, an oligonucleotide probe or a PCR primer is

synthesized. However, it is generally difficult to purify a protein. On the other hand, a method in which nucleic acid information is utilized is one in which the objective gene is isolated, by utilizing gene homology among organisms, by using a gene of the other organism as a probe, and by using the hybridization method. The gene homology among organisms varies depending on the organism used and the gene used, and the condition of the hybridization is experimentally determined after carrying out under various conditions. It can be a case that the best condition can not be determined. Further, in these methods, it is necessary to confirm the biological activity of the obtained gene product by using some other methods.

On the other hand, the genetical method is a method in which the gene encoding a protein with tha same function derived from a different organism is introduced into a mutated microorganism in which the objective gene is genetically deleted, such as *E. coli*, *B.subtilis* and yeast *etc.*, in such a manner that it can be expressed in the organism, and the growth is complemented. This method is also called as the genetically complementary method, the biological activity of the gene product is already confirmed, at time of isolating the gene. However, when a gene derived from a higher organism is expressed in a microorganism, there is a case that the growth is not complemented, by the reason that the modification, for example the addition of a sugar chain, is not achieved and can not perform the enzyme activity, or the enzyme activity can not be achieved because that the protein derived from the vector or transit peptide is added to the N-terminal. Nevertheless, in the tetrapyrrole system in a plant, Glu-tRNA Reductase which synthesizes Glutamate-1-semialdehyde from Glu-tRNA, and Glutamate-1-semialdehyde aminotansferase which synthesizes 5-Aminolevulinic acid from Glutamate-1-semialdehyde, are cloned by the genetically complementation method, respectively by using *E. coli hemA* gene deleted mutant strain and *hemL* gene deleted mutant strain. (IIag, L. L. *et al.* , (1994) *Plant Cell* 6:265-275). Thus, it can be thought to be effective to use a gene deleted mutant strain of *E. coli*. in isolating plant protoporphyrinogen oxidase. As an *E. coli* mutant strain in which protoporphyrinogen oxidase is deleted, SASX38 (Sasarman, A. *et al.* (1979) *J. Gen. Microbiol.* 113:297-303) and VSR751 (Nishimura, K. *et al.* (1995) *J. Bio. Chem.* 270:8076-8080), a mutant strain in which *hemG* gene is deleted, is known.

5. Preparation method for cDNA library

Total RNA of a plant can be extracted from a plant body, by using known methods, for example, guanidine method,

guanidine phenol method, SDS phenol method, *etc.* Further, mRNA can be purified by conventional methods, for example, oligo dT cellulose method, *etc.*

The preparation of cDNA can be performed according to the conventional method as described in the above literatures. The double stranded cDNA can be synthesized using the purified mRNA as a template by Okayama-Berg method, Gubler-Hoffman method *etc.*

The cDNA library can be prepared according to the conventional method, by ligating the cDNA with a plasmid or λ phage *etc.* It is preferable that a vector which has a promoter, such as, λ phage lacZ, tac, at upstream of MCS, is selected (for example, pUC series plasmid and λgt11 series phage *etc.*), and to prepare an expression type cDNA library. Further, unidirectional library can be prepared by using a primer-adaptor *etc.* as a primer.

6. Cloning and analysis of cDNA of protoporphyrinogen oxidase

The protoporphyrinogen oxidase gene can be cloned, by introducing the expression type cDNA library into an *E. coli* mutant strain in which *hemG* (protoporphyrinogen oxidase gene) is deleted, and isolating a cDNA clone which complements the growth. The whole DNA sequence of the gene thus cloned can be determined by Maxiam-Gilbert method or Sanger method. Further, the search for protein codign region, and the analysis of homology of the nucleotide sequence with known genes can be performed by using a commercially available software for analysis of a nucleotide sequence, for example, Genetyx (SDC Co. Ltd.,) or DNASIS (Hitachi software engineering Co., Ltd.).

7. Cloning of the gene of protoporphyrinogen oxidase tolerant to photobleaching herbicide from a callus tolerant to photobleaching herbicide

In case that the protoporphyrinogen oxidase gene is cloned from a tobacco callus having a tolerance mechanism to photobleaching herbicide, caused from the conversion of protoporphyrinogen oxidase into a tolerant type to a photobleaching herbicide, the construction of cDNA library and the genetically complementary method is not necessarily needed, the cloning is relatively easily performed by PCR. The cloning of the protoporphyrinogen oxidase gene having a biological activity and the subcloning into a plasmid vector can relatively easily be conducted by designing a PCR primer which can amplify the whole open reading frame encoding protoporphyrinogen oxidase, from the nucleotide sequence information of the tobacco chloroplast type protoporphyrinogen oxidase gene disclosed by the present invention; then by

conducting RT-PCR (mRNA is converted to cDNA by a reverse transcriptase, and PCR thereof is performed.) by using these primers.

The extent of tolerance of thus obtained gene is relatively easily examined by introducing the obtained plasmid into the mutant strain of *E. coli* having a deletion of *hemG*, and by comparing the extent of inhibition of growth of the obtained *E. coli* by the photobleaching herbicide.

8. Production of a plant tolerant to photobleaching herbicides

By using the protoporphyrinogen oxidase gene which is tolerant to a photobleaching herbicide disclosed by the present invention, a plant which is tolerant to photobleaching herbicides can be produced. That is, a transformed plant can be produced by incorporating into a appropriate plasmid vector an expression cassette comprising a promoter capable of working in a plant cell, the protoporphyrinogen oxidase gene which is tolerant to photobleaching herbicides, and a terminator capable of working in a plant cell; by incorporating the expression cassette using known gene incorporation methods, such as, the *Agrobacterium* method, the electroporation method into a protoplast and the particle gun method, *etc*. Thus obtained plant has an agriculturally useful character of the tolerance to photobleaching herbicides. Further, when a expression cassette which has a different gene is used simultaneously, the nature to be tolerant to photobleaching herbicides can be utilized as a selected marker of the transformed plant.

As the appropriate plasmid vector, pBI plasmid or pMON plasmid can be listed in case of using the *Agrobacterium* method, and pUC plasmid and pBluescript plasmid can be listed in case of using the other method. Further, as the promoter capable of work in a plant, a cauliflower mosaic virus (CaMV) 35S promoter can be listed, and as the terminator capable of working in a plant, a CaMV terminator can be listed. Detailed explanation is described in the above-described experimental text books, such as Plant Molecular Biology manual, Gelvin, S. A. *et al.* , Kluwer Academic Publishers, 1991, 1995 (Second edition) *etc.*

Examples

Hereinafter the present invention is described in more detail by showing examples, but the present invention is not limited to these examples.

Example 1 Selection of tobacco callus tolerant to photobleaching herbicide.

A. Preparation of tobacco callus and regeneration of the callus and study of sensitivity to photobleaching herbicide

Tobacco (*Nicotiana tabacum* CV. Xanthi NC) was grown to 40cm tall in a green house. And a leaf was cut, and the leaf was cut to square-shape of 5cm width by a knife, then immersed in 70 % ethanol for 30 seconds and in an aqueous solution of sodium hypochlorite (the effective concentration of chlorine was 1 %) for 15 minutes to conduct surface-sterilization. After washing it for three times by sterilized water and removal of epidermis, the leaf fragment was suspended in 0.6M manitol (pH 5.8) containing an enzyme solution (1% cellulase onozuka R-10 and 0.05% macerozyme R-10 (manufactured by Yakult Co., Ltd.)) at 26 ℃ for three hours. After the enzyme treatment, protoplasts were washed with centrifigation by Murasige-Skoog (MS) medium for three times, and the obtained protoplast was suspended into MS solid medium containing 0.6% agar and 1ppm of NAA and BA, to make a cell number of 10^8/ml, and pored into a dish at 1cm thick. The protoplast was cultured at 27 ℃ under darkness to regenerate a callus. Four weeks after the incubation, the agar medium containing a callus grown up to about 1mm large, was cut to rectangular shape having 1cm width, and put on MS solid medium containing various concentration of the compound H (4-chloro-3-[2,4-dichloro-5(2-propenyloxy)phenyl]-5-difluoromethoxy-1-methyl-1H-pyrazole, described in Toku Kai Hei 3-163063), and the sensitivity of tobacco callus to photobleaching herbicide was examined. As shoen in Table 1, it became apparent that the callus completely dies at 10nM.

Table 1 Sensitivity of tobacco callus to the compound H

Conc.(nM)	Survival Rate (%)
0	100
2.5	30
5	10
10	0
20	0
40	0
8	0

B. Selection of callus highly resistant to photobleaching herbicide

Using a medium containing the compound H containing two times concentration (20nM) of the concentration at which tobacco callus completely dies, the selection of tobacco callus tolerant to photobleaching herbicide has been started. $1X10^5$ of tobacco callus have been selected (Table 2), in the course of re-generation from $1X10^{10}$ of the protoplast. Thus obtained resistant callus was transplanted into media having an increasing concentration of compound H, and thirty six cell lines which can be grown at even 2,400 nM were obtained finally, and three cell lines (ETR-056, RTR-245 and ETR-253), growth of which were especially superior were selected.

Table 2
Selection course of a strain tolerant to the photobleaching herbicide

Conc.(nM)	Number of resistant cell lines
20	100,000
30	50,000
50	40,000
75	7,200
150	254
300	120
600	66
1200	46
2,400	36
5,000	0

number of protoplast given:$1X10^{10}$

Example 2. Analysis of the resistant mechanism of the callus tolerant to photobleaching herbicide.

A. Accumulation of protoporphyline IX in photobleaching herbicide resistant cell lines

The present inventors have tried to analyze the resistant mechanism in the resistant strain tolerant to photobleaching herbicide. It has been reported that in a plant treated by photobleaching herbicide, protoporphyrin IX is accumulated, the present inventors tried to measure the accumulation of protoporphyrin IX periodically. A resistant callus which has been subcultured in MS liquid medium containing 1,200 nM of compound H under light condition was cultured for 24 hours in a dark condition, then transplanted into MS liquid medium containing 1,200 nM of compound H, and after 6, 12, 18, 24, 32 and 48 hours later, the callus was collected, and

protoporphyrin IX was measured. The extraction and assay of the protoporphyrin IX from the callus were conducted as follows (Matringe, M. *et al.*(1989) *Biochem, J.* 260:231-235.). That is, 500mg (wet weight) of the callus was homogenized in a mortar, and extracted with 7.5ml of acetone: 1N NH_4OH (9:1). The extract was centrifuged at 3,000xg for 10 minutes, then the supernatant was collected. To the supernatant 2.5 ml of hexane was added and mixed sufficiently, then, the lower layer was recovered. Further, after washing with hexane two twice, the amount of protoporphyrin IX obtained in the lower layer was measured from the value of Ex 405nm-Em 631.5nm by a fluorescence spectrophotometer. All the extraction procedures were conducted under the safety light.

As a result, the amount of protoporphyrin IX was 1.3μg / g fresh weight at 24 hours and 1.9μg / g fresh weight at 48 hours for sensitive cell line, and 1.7μg / g fresh weight at 24 hours and 4.6μg / g fresh weight at 48 hours for ETR-056 line, that is, the accumulation of protoporphyrin IX was observed. On the other hand, in ETR-245 and ETR-253 strain, little accumulation of protoporphyrin IX was recognized.

B. Cross-resistance of a cell lines tolerant to photobleaching herbicide against various herbicides.

Further, cross-resistance of a resistant strain tolerant to herbicides with various modes of action was examined. Sensitivity to fomesafen (trade mark: FLEX) and oxadiazon (trade mark: RONSTAR), which are photobleaching herbicide and accumulate protoporphyrin IX; pyrazoxyfen (trademark: PAICER) which is a chlorophyll synthesis inhibitor and causes bleaching; DPX-84 and bialaphos (trademark: BASTA) and glyphosate (trademark: ROUNDUP) which are an inhibitor of the amino acid synthesis; butachlor (trademark: MACHETE) which is a protein synthesis inhibitor; propanil (trademark: STAM) which is an inhibitor of light synthesis photochemical system II and paraquat (trademark: PREEGLOX) which is an inhibitor of photosystem I and produces an active oxygen was compared. Into MS agar medium containing each herbicides, each resistant strain was transplanted and minimum inhibition concentration (MIC) was measured. As a result (Table 3), each resistant cell line showed strong resistance to fomesafen and oxadiazon which are an inhibitor of protoporphyrinogen oxidase inhibitor and accumulate protoporphyrin IX. Further, ETR-056 showed resistance to paraquat, and ETR-253 showed resistance to pyrazoxyfen, DPX-84 and glyphosate. From the above results, ETR-056 was thought to have resistance to an active oxygen caused by overproduction of superoxide dismutase, and ETR-253 was thought to have resistance to many herbicides caused by decreasing of a membrane permeability, and ETR-245 was thought

to have resistance caused by mutation of a target enzyme or overproduction of a target enzyme.

Table 3 Sensitivity of photobleaching herbicide resistant celllines to various herbicides

	MIC(ppm)			
Herbicide	Sensitive strain	ETR-056	ETR-245	ETR-253
fomesafen	0.4	4	4	4
oxadiazon	1	1000	1000	1000
butachlor	100	100	100	100
pyrazoxyfen	100	100	100	200
propanil	100	100	100	100
bialaphos	10	1	1	10
DPX-84	0.01	0.01	0.01	0.1
glyphosate	230	230	230	1000
paraquat	10	100	10	10

C. Sensitivity of photobleaching herbicide resistant cell lines to the other ET herbicides

MIC of compound A to the sensitive cell lines, ETR-056, ETR-245 and ETR-253 were measured. As the result, MIC to the sensitive cell line was 5 nM, but, MIC to the resistant cell lines were over 2,400 nM, and becomes apparent that the resistant cell lines have strong resistance to the compound A. Therefore, in the following examples, the compound A was used as a photobleaching herbicide and ETR-245 was used as a resistant strain.

D. Extraction of crude enzyme preparation from sensitive cell and ETR-245 cell line

Using the same procedures described in Nicolaus, B. *et al.* (1993) in "Target Assay for Modern Herbicide and Related Phytotoxic Compound" Lewis Publishers, pp.35-41, five volume of extraction buffer (50 mM Tris-HCl (pH 7.5), 0.5 M sucrose, 0.2 %BSA, 1 mM EDTA) was added to callus which was cultured in MS solid medium for one month, and homogenized. After filtration with gauze, filtrate was centrifuged with 10,000xg at 4 °C for 5 minutes. Thus obtained precipitate was suspended with 25 ml of ethanol buffer, centrifuged with 150xg at 4 °C for 2 minutes. Thus obtained supernatant was centrifuged with

4,000xg at 4 °C for 15 minutes, and the precipitate was dissolved with 2ml of 20% glycerol. The protein amount in the obtained crude enzyme preparation was measured with the protein measuring kit (manufactured by Bio-Rad Co., Ltd.).

E. Preparation of protoporphyrinogen IX

The powder of sodium amalgam was prpared as follows. Into a round flask of 500ml volume, 13g of mercury and 0.5g of thin-layer metal sodium were added, and equilibrated with nitrogen gas for 5 minutes, and it was shaked for 5 minutesthen. 8.4 g of protoporphyrin IX was dissolved into 10 mM KOH containing 15 ml of 20% ethanol, incubated at 4 °C. To 4ml of the protoporphyrin IX solution, equal amount of reaction mixture (0.1 M MES, 50 mM ascorbic acid) was added, and 16 mg of sodium amalgam freshly prepared was added, and shaken vigorously under nitrogen gas in a dark. The reaction mixture was filtered with a three-layer glass filter in a dark, and diluted 2.5 times with 0.1 M MES (pH4.5), and divided into 1 ml each in a light-shielded test tube and stored at -80 °C.

F. Measurements of protoporphyrinogen oxidase activity

3 ml of reaction buffer (100 mM Tris-HCl (pH 7.6), 1 mM EDTA, 5 mM DTT, 0.03% Tween 80) were added to a cell of a fluorescence spectrophotometer, then, the crude enzyme prparation was added at the final concentration of 0.1 mg protein/ml, and it was heated gradually to room temperature. After heating, protoporphyrinogen IX was added at the final concentration of 2 μM, and the gaseous phase was replaced with nitrogen gas, and a reaction was started. Fluorescence was monitored with a spectrophotometer for 30 minutes starting 10 minutes after the start of the reaction, and enzyme activity was measured (Ex:405nm,Em:631.5nm).

G. Inhibitory effect of compound A on protoporphyrinogen oxidase preparation extracted from ETR-245 cell

Activity of protoporphyrinogen oxidase in crude enzyme preparation which was extracted from a sensitive tobacco and ETR-245 and the extent of inhibition by compound A were compared (Table 4). As a result, activity of the crude enzyme preparation derived from the sensitive strain was 2.39 units, and the activity of the crude enzyme liquid derived from ETR-245 strain was 2.85 units, and there was no large difference between the activities. On the other hand, 50% inhibition concentration (IC_{50}) value of compound A to the activity of protoporphyrinogen oxidase extracted from the sensitive cell

line was 48 nM, on the other hand, IC_{50} value of compound A to the enzyme activity extracted from ETR-245 was 5,000 nM, which showed the resistance of more than 100 times at enzyme level. Incidentally, since the reaction mixture became turbidat the concentration of more than 5,000 nM, it was impossible to measure the fluorescence. From the above results, the resistant mechanism in ETR-245 to the photobleaching herbicide could be thought that the some mutations in the structure of the enzyme occurred, and was not over production of protoporphyrinogen oxidase which was a target enzyme of photobleaching herbicide.

Table 4. Inhibitory effect of compound A on the protoporphyrinogen oxidase activity from sensitive and ETR-245 cell line

	Enzyme activity(unit)	
Concentration of Compound A (nM)	Sensitive	ETR-245
0	2.39	2.85
10	4.32	3.59
20	3.62	-
40	1.84	4.41
100	0.00	2.21
500	0.00	3.49
1,000	0.00	2.85
2,400	0.00	2.48
5,000	0.00	1.56
IC_{50} value(nM)	48	)5000

1 unit= 1nM protoporphyrin IX/min/mg protein

Example 3. Cloning of tobacco protoporphyrinogen oxidase cDNA

A. Preparation of mRNA

9g of a green leaf of tobacco (Nicotiana tabacum CV. SR1) was cut, and it was homogenized in liquid nitrogen. To the homogenate, 40 ml of RNA extraction buffer (200 mM Tris-HCl (pH9.0), 100 mM NaCl, 10 mM EDTA, 0.5% SDS, 0.1% 2-ME) and 40 ml of Tris-saturated phenol were added, and shaken vigorously for 10 minutes, then centrifuged with 2,000xg for 10 minutes, and the supernatant was recovered. To the supernatant, equal

amount of Tris-saturated phenol was added, and shaking for 10 minutes and centrifugation for 10 minutes were repeated. To the supernatant thus derived, equal amount of phenol: chloroform: isoamylalcohol (25:24:1) were added, and shaking for 10 minutes and centrifugation for 10 minutes were further repeated two times. To the supernatant thus derived, equal amount of chloroform: isoamylalcohol (24:1) were added, and shaking for 10 minutes and centrifugation for 3 minutes were conducted two times. To the supernatant thus derived, 1/10 amount of 3 M sodium citric acid (pH5.2) and 2.5 fold volume of ethanol were added, incubated for 30 minutes at -80 °C. Then, after centrifugation at 2,000xg for 20 minutes at 4 °C, the precipitate was washed with 70% ethanol. To the dried precipitation, distilled water was added at the final concentration of 1mg/ml, and 10 M lithium chloride was added at the final concentration of 2 M. Fter incubation on ice for 2 hours, the precipitate was recovered with 2,000xg 30 minutes at 4 °C, and total RNA fraction was obtained.

Purification of mRNA from the total RNA fraction was conducted by using origo dT span column (manufactured by Pharmacia), according to the attached manual.

B. Preparation of cDNA library

Preparation of cDNA library was conducted by using Superscript Lambda System (manufactured by GIBCO BRL Co., Ltd.). By using this kit, cDNA can be inserted at the positive orientation against lacZ promoter of λgt22A, by using a primer-adaptor at the first strain synthesis.

4 μg of mRNA was used as a template for cDNA sunthesis. cDNA first strand synthesis, cDNA second strand synthesis, ligation of adaptor, restriction enzyme digestion and column chromatography were conducted according to the attached manual. cDNA thus obtained was ligated with λgt22A, and phage particle was reconstructed using Gigapack Gold (manufactured by Stragene Co., Ltd.), according to the attached manual. The primary library containing about one million and nine hundred thousand of independent clones were infected with *E. coli* Y1090 (r-) strain, multiplies and stored as an amplification library.

C. Genetic complementation

Using an *E. coli* SASX38 strain having deletion of *hemG* (protoporphyrinogen oxidase) gene, and by using the method of Nishimura (Nishimura, K. *et al.* (1995) *J. Biol. Chem.* 270:8076-8080), genetic complementation was conducted. To the SASX38 strain which had been pre-cultured in LB liquid medium containing 10 mM MgSO4 and 02% maltose, the above-mentioned

amplification library was infected and spread on LB solid medium, then gene expression was induced. After it was cultured for two days at 37 °C, some large colonies with recovered growth were observed, among many small colonies. The large colony was cultured in LB liquid medium over night, then, chloroform was added at the final concentration of 4%, mixed sufficiently, incubated over 30 minutes at room temperature and centrifuged to obtain phage particles as supernatant. The phage particles thus obtained were again infected to *E. coli* Y1090(r-) stain, and a single plaque was suspended in SM medium containing 4% chloroform. The recombinant phage particle recovered was infected again to the SASX38 strain, and the phage which conferred restorotion of poor growth of the *E. coli* was reselected. The expression of protoporphyrinogen oxidase activity in *E.coli* by these recombinant phage vector was confirmed by the above-mentioned method.

D. Analysis of cDNA insert

Amplification of cDNA insert was conducted by PCR. That is, using total DNA in the recovered recombinant phage particle as a template, Taq DNA polymerase (TAKARA EX Taq, manufactured by Takara Shuzo Co., Ltd.), a forward primer (5'ATT GGT GGC GAC GAC TCC TGG AG-3', SEQ ID No. 4) and a reverse primer (5'-CCA GAC CAA CTG GTA ATG GTA GCG-3', SEQ ID No. 5) for λgt22A, 30 cycles of PCR were conducted in which one cycle comprises 94 °C for 1.5 minutes, 69 °C for 1.5 minutes and 72 °C for 2 minutes. A part of reaction product was analysed by agarose gel electrophoresis, and it became clear that around 2.0 kbp cDNA was amplified in all samples. The amplified cDNA was recovered by chloroform treatment and ethanol precipitation, then compared by a restriction enzyme digestion analysis, indicating that *Eco*RV site was observed in each cDNA.

E. Subcloning of cDNA insert and confirmation of biological activity

The largest cDNA insert among cDNA inserts amplified by PCR was inserted at the positive orientation against the lacZ promoter into *Eco*RV site of pBluescript SK(-), by using TA cloning method described in the above mentioned literatures (for example, Current Protocols in Molecular Biology, John Wiley & sons, Ausubel, M. *et al.*). That is, the pBluescript SK (-) was digested by *Eco*RV and recovered, and Taq DNA polymerase (TAKARA EX Taq, manufactured by Takara Shuzo Co., Ltd.) was added and reacted at 75 °C for 2 hours in the presence of dTTP to add T to 3' end of the plasmid DNA. After

the addition of T, the plasmid recovered and the largest cDNA insert recovered after PCR were ligated using DNA Ligation Kit (manufactured by Takara Shuzo Co., Ltd.), according to the attached explanation. With thus obtained reaction mixture, competent cells of XL-1 Blue strain (manufactured by Stratagene Co., Ltd.) produced by the $CaCl_2$ method was transformed and spread on LB agar medium containing IPTG and ampicillin, and cultured at 37 °C for overnight. After the incubation, some white colonies with cDNA insert were selected, and cultured in LB liquid medium containing ampicillin for overnight, then plasmid DNA was prepared by the alkaline lysis method. Thus obtained plasmid DNA was digested with a restriction enzyme, and *E. coli* having a plasmid in which cDNA was inserted at the positive orientation of the promotor, was selected. Thus obtained plasmid was named as pBNtPX-1 (Figure 1), and glycerol was added at the final concentration of 15 % to the *E. coli* culture medium and stored at -80 °C.

The *E. coli* having pBNtPX-1 was cultured in LB liquid medium containing ampicillin for overnight, then the plasmid was purified in large quantity by the Cesium chloride method. Using the obtained plasmid, the competent cell of *E. coli* SAX38 strain produced by the $CaCl_2$ method was transformed. The transformed SASX38 strain was spread on LB agar medium, and cultured for overnight at 28°C. As a result, in case that pBNtPX-1 was used, many large colonies with recovered growth were observed among small colonies. As described in the above, protoporphyrinogen oxidase activity in *E.coli* by pBNtPX-1 was confirmed.

F. Analysis of nucleotide sequence of cDNA

From the pBNtPX-1 which recovered the poor growth of *E. coli* SASX38 strain, deletion clones were produced by using Deletion Kit for Kilo-Sequence (manufactured by Takara Shuzo Co., Ltd.), according to the attached manual, and the nucleotide sequence and amino acid sequence in the open reading frame were analyzed by using Cycle Sequencing Kit AmpliTaq FS (manufactured by Perkin-Elmer Corporation) and an auto sequencer (manufactured by ABI PRISM 310, Perkin-Elmer Corporation) according to the attached manual. The obtained nucleotide sequence and the amino acid sequence in the open reading frame are shown in the SEQ ID No. 1. By using Genetyx (SDC Co., Ltd.), gene analysis software, nucleotide sequence and amino acid sequence were compared with those of the protoporphyrinogen oxidase of *Arabidopsis thaliana* which have already reported (WO 95/34659), and the homology was examined. As a result, pBNtPX-1 showed high homology of 69 % at nucleotide level and 76 % at amino acid level with the

chloroplast-type protoporphyrinogen oxidase cDNA of *Arabidopsis thaliana*, and considered to be a chloroplast-type protoporphyrinogen oxidase cDNA of tobacco.

Further, the polypeptide was confirmed to be the protoporphyrinogen oxidase, from the fact that it has a dinucleotide binding domain (GXGXXG) (Nishimura, K. *et al.* (1995) *J. Biol. Chem.* 270:8076-8080), which is conserved in the N-terminal of protoporphyrinogen oxidase of many organisms. Further, in protoporphyrinogen oxidase of *B.subtilis* and animal, only from 8 to 11 amino acids were found at up-stream of the domain, on the contrary, 77 amino acids were found in a chloroplast-type protoporphyrinogen oxidase gene of tobacco.

Table 5 Comparison of amino acid sequence of tobacco (upper column) and *Arabidopsis thaliana* (lower column) of a chloroplast-type protoporphyrinogen oxidase

Homology: 75.9%

```
   1' MTTTPIANHPNIFTHQSSSSPLAFLNRTSFIPFSSISKRN-SVN-CNGWRTRCSVAKDYT
                        *..*..*.   ..*.**.* ..* ..  * ***** . *
   1"                  MELSLLRPTTQSLLPSFSKPNLRLNVYKPLRLRCSVAGGPT

  59' VPSSAVDGGPAAEL--DCVIVGAGISGLCIAQVMSANY----PNLMVTEARDRAGGNITT
      * ** ..** ....  ******.*********.....     ***.****.**.****.*
  42" VGSSKIEGGGGTTITTDCVIVGGGISGLCIAQALATKHPDAAPNLIVTEAKDRVGGNIIT

 113' VERDGYLWEEGPNSFQPSDPMLTMAVDCGLKDDLVLGDPNAPRFVLWKGKLRPVPSKLTD
       * .*.******************.**.***********.*******.************
 102" REENGFLWEEGPNSFQPSDPMLTMVVDSGLKDDLVLGDPTAPRFVLWNGKLRPVPSKLTD

 173' LPFFDLMSIPGKLRAGFGAIGLRPSPPGHEESVEQFVRRNLGGEVFERLIEPFCSGVYAG
      ********* **.******.*.******.*****.*******.*****************
 162" LPFFDLMSIGGKIRAGFGALGIRPSPPGREESVEEFVRRNLGDEVFERLIEPFCSGVYAG

 233' DPSKLSMKAAFGKVWKLEETGGSIIGGTFKAIKERSSTPKAPRDPRLPKPKGQTVGSFRK
      ******************..***********.**...*** ********.*********
 222" DPSKLSMKAAFGKVWKLEQNGGSIIGGTFKAIQERKNAPKAERDPRLPKPQGQTVGSFRK

 293' GLRMLPDAISARLGSKLKLSWKLSSITKSEKGGYHLTYETPEGVVSLQSRSIVMTVPSYV
      ******.*********.*******.*** *.***.******.*.**.**.*.******.*
 282" GLRMLPEAISARLGSKVKLSWKLSGITKLESGGYNLTYETPDGLVSVQSKSVVMTVPSHV

 353' ASNILRPLSVAAADALSNFYYPPVGAVTISYPQEAIRDERLVDGELKGFGQLHPRTQGVE
      **..***** .**.***..*****.**.****.****.* *.******************
 342" ASGLLRPLSESAANALSKLYYPPVAAVSISYPKEAIRTECLIDGELKGFGQLHPRTQGVE

 413' TLGTIYSSSLFPNRAPKGRVLLLNYIGGAKNPEILSKTESQLVEVVDRDLRKMLIKPKAQ
      **************** **.********..*..****.*..***.************..
 402" TLGTIYSSSLFPNRAPPGRILLLNYIGGSTNTGILSKSEGELVEAVDRDLRKMLIKPNST

 473' DPLVVGVRVWPQAIPQFLVGHLDTLSTAKAAMNDNGLEGLFLGGNYVSGVALGRCVEGAY
      *** .****************.*.*.***.....* **********.************
 462" DPLKLGVRVWPQAIPQFLVGHFDILDTAKSSLTSSGYEGLFLGGNYVAGVALGRCVEGAY
```

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533' EVASEVTGFLSRYAYK   548
     *.* **..*.******
522" ETAIEVNNFMSRYAYK   537
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Example 5 protoporphyrinogen oxidase cDNA of tobacco callus tolerant to photobleaching herbicide

A. Cloning by PCR

After extracting the total RNA by the SDS/phenol method from photobleaching herbicide tolerant callus, ETR-245 cell line, and sensitive cell line, respectively, mRNA was purified by mRNA purification Kit (manufactured by Pharmacia Co., Ltd.). Reverse transcription reaction was conducted using 1 μg of the purified mRNA as a template and using Oligo(dT)12-18 (manufactured by Life Technology Oriental Inc.) as a primer and using Superscript™ RNaseH-Reverse Transcriptase (manufactured by Life Technology Oriental Inc.) according to the attached manual at 37 °C for 1 hour, and then cDNA was purified by phenol/chloroform extraction and ethanol precipitation. Protoporphyrinogen oxidase cDNA was attempted to be amplified by PCR, using the obtained cDNA as a template. A forward primer (5'-GCG GTC TAC AAG TCA GGC AGT CAT-3', SEQ ID No.6) and a reverse primer(5'-CAT GCC AAT TTT CCC AAG GCA TGA TCG TAT T-3', SEQ ID No.7) were used as the primer for PCR. One cycle of 94 °C for 3 minutes, and 30 cycles of 94 °C for 20 seconds, 61 °C for 30 seconds and 72 °C for 1 minute and 30 seconds and one cycle of 72 °C for 5 minutes were conducted by using a Taq DNA polymerase (manufactured by TAKARA EX Taq, Takara Shuzo Co., Ltd.) in a thin wall tube. A part of the obtained PCR reaction mixture was analyzed by agarose gel electrophoresis, DNA fragment having about 1.7 kbp was observed. These can be thought to be protoporphyrinogen oxidase cDNA fragment. Accordingly, these DNA was ligated with the plasmid vector pCR™2.1 having T over hang, using Original TA Cloning KIT (manufactured by Invitrogene Co., Ltd.), according to the attached manual. And, with a ligation mixture, the competent cell of *E. coli*, XL-1 Blue strain (manufactured by Stratagene Co., Ltd.) prepared by the $CaCl_2$ method, was transformed, and spread on LB agar medium containing IPTG, Xgal and ampicillin, and incubated at 37 °C for overnight. After the incubation, some white colonies with cDNA insert were selected, cultured with shaking in LB liquid medium containing ampicillin for overnight, then plasmid DNA

was prepared by the alkaline lysis method. The obtained plasmid DNA was digested with restriction enzyme, and *E. coli* having a plasmid in which each cDNA was inserted at the positive orientation against the promotor was selected. Plasmids in which chlorophyll type protoporphyrinogen oxidase cDNA were inserted were named as pCR-HC or pCR-RC (Fig. 2), respectively. Thne, glycerol was added to the final concentration of 15%, and the *E. coli* was stored at -80 °C.

The *E. coli* having each of these two kind of plasmid were cultured in LB liquid medium containing ampicillin for overnight, then large quantity of plasmid wsa purified by the Cesium chloride method. Competent cells of *E. coli* SASX38 strain produced by the $CaCl_2$ method was transformed with the obtained plasmid. The transformed SASX38 strain was spread on LB agar medium and incubated at 28°C for overnight. As a result, in any case that any incubated plasmid was used, many large colonies whose growth was recovered were observed among small colonies. As described, expression of the protoporphyrinogen oxidase activity in *E. coli* by pCR-HC and pCR-RC was confirmed.

B. Comparison of resistance of each gene product to compound A

The *E. coli* SASX38 strain containing either of the above-described two kinds of plasmid was cultured with shaking in LB liquid medium containing 50 μg /ml of ampicillin at 28°C for overnight. After measuring of OD_{600} value, the culture was added into the LB liquid medium containing 50 μg /ml of ampicillin and 0, 1, 10, 100, 1000, 2,000, 5,000 or 10,000 nM of compound A, so as to become same OD_{600} value, and were cultured with shaking at 28°C. A part was collected periodically, OD_{530} value were measured by a micro plate reader (MTP-120, manufactured by Corona Electric Co., Ltd.), and the concentration at which the growth of *E. coli* of control (Compound A 0nM) was inhibited by 50% (IC_{50}) was calculated. As a result, the IC_{50} value in the *E. coli* containing pCR-HC or pCR-RC was about 2.5 nM or 10,000 nM or more, respectively and R/S ratio (IC_{50} value of a resistant type / IC_{50} value in a wild type) was over 4,000. Incidentally, since the medium became turbid at the concentration of 10,000nM or more, it was impossible to measure OD_{530} value. From the above result, the resistant mechanism to Compound A of protoporphyrinogen oxidase in ETR-245 was thought that some mutation occurred in chloroplast-type protoporphyrinogen oxidase gene, and the enzyme was converted to a photobleaching herbicide resistant type.

C. Comparison of the nucleotide sequence of the chloroplast type protoporphyrinogen oxidase cDNA

Comparison of the entire nucleotide sequence of the protoporphyrinogen oxidase cDNA derived from the sensitive and

ETR-245 cell line, contained in pCR-HC or pCR-RC, respectively was performed. From the nucleotide sequence information of the cDNA derived from tobacco leaf, a primer set for primer-walking was synthesized, and entire nucleotide sequence of the both cDNA was compared. As a result, the nucleotide sequence of pCR-HC was quite the same sequence as that of protoporphyrinogen oxidase cDNA derived from the tobacco leaf, but, in the pCR-RC, a mutation was observed in only one point. That is, C was mutated to T at 717th nucleotide in SEQ ID No. 1, as the result, alanine was mutated to valine at 231th amino acid in SEQ ID No.1. Amino acid sequence of the protoporphyrinogen oxidase resistant to the photobleaching herbicide is shown in SEQ ID No. 2, and the nucleotide sequence of the cDNA is shown in SEQ ID No.3. The homology of tobacco and *Arabidopsis thaliana* protoporphyrinogen oxidase amino acid sequence was high (Table 5), this mutation was almost the same mutation as in the mutation to the photobleaching herbicide resistant type (pArac-1Val), accompanied by the mutation from alanine of 220th amino acid to valine in *Arabidopsis thaliana* protoporphyrinogen oxidase, described in WO/95/34659.

Example 6.Photobleaching herbicide resistant type cDNA of *Arabidopsis thaliana*

A. Cloning of chloroplast-type protoporphyrinogen oxidase cDNA of *Arabidopsis thaliana* and conversion to the photobleaching herbicide resistant type

The cloning of chloroplast-type protoporphyrinogen oxidase gene was conducted by preparing cDNA library and genetic complementation method in which *E.coli* mutant strain SAS38 defective *HemG*, similarly in the case of tobacco. Sub-cloning of the cDNA thus obtained to *Eco*RV site of pBluescript SK(-) was conducted by using the TA coning method.

A plasmid showing a bioactivity in which the protoporphyrinogen oxidase gene was inserted at the positive orientation against the promotor was named as pBAtPX-C (Fig. 3). The entire nucleotide sequence of the plasmid was confirmed to be entirely the same as the known sequence (W/O 95/34659), by the primer walking using a primer designed from the sequence.

This plasmid was used for the production of a protoporphyrinogen oxidase cDNA resistant to photobleaching herbicide, by the mutation of cDNA valine from alanine at 220th amino acid, as described in WO 95/34659. Single strand DNA was prepared from *E. coli* XL-1 Blue containing pBAtPX-C by using a helper phage VCS-M13 (manufactured by Stratagene Co., Ltd.). Then, using a kit for site-specific mutagenesis (manufactured by Mutan-K, Takara Shuzo Co., Ltd.) in which the Kunkel method was utilized and oligonucleotide for the introduction (5'-GGT GTT TAT GTT GGT GAT CC-3') (SEQ ID No.

8), the cDNA was converted to photobleaching herbicide resistant type. In order to avoid non-specific mutation in the course of site-specific mutagenesis, resubcloning of the cDNA in the plasmid thus obtained into pBluescript SK(-) was again conducted. Further, the entire nucleotide sequence of the cDNA was determined and it was confirmed to be the objective nucleotide sequence. The final plasmid which was tolerant to the light requiring herbicide was named as pBAtPX-RC (Figure 3).

B. Comparison of resistance to Compound A of two type of protoporphyrinogen oxidase from *Arabidopsis thaliana*

Comparison of the resistance to the Compound A of wild type protoporphyrinogen oxidase gene product encoded by pBAtPX-C and resistant type gene product encoded by pBAtPX-RC was attempted. Using the same method as described in the comparison of resistance to the Compound A of each gene product in Example 5B, IC_{50} value of each protoporphyrinogen oxidase to the Compound A was measured, respectively. As the result, IC_{50} value was about 5 nM in case of the wild type, and 1,500 nM in the resistant type, and R/S ratio (IC_{50} value of the resistant type/ IC_{50} value of the wild type) was about 300. R/S ratio in the case of tobacco was over 4,000, therefore, the R/S ratio in the case of tobacco was found to be over ten times higher, comparing with the *Arabidopsis thaliana*. This difference was highly unexpected result, when the high homology of the amino acid sequence between both protoporphyrinogen oxidase was taken into consideration.

Example 7. Resistance to several compounds

By using the *E. coli* SASX38 strain having pCR-HC with tobacco wild type protoporphyrinogen oxidase cDNA, the *E. coli* SASX38 strain having pCR-RC with tobacco resistant type protoporphyrinogen oxidase gene, the *E. coli* SASX38 strain having pBAtPX-C with *Arabidopsis thaliana* wild type protoporphyrinogen oxidase gene and the *E. coli* SASX38 strain having pBAtPX-RC with *Arabidopsis thaliana* resistant type protoporphyrinogen oxidase cDNA, IC_{50} value of several pyrazole compound (Compound B, C, D, E, F and G) to the growth of each *E. coli* was measured, and R/S ratio (IC_{50} values of the resistant type/ IC_{50} value of the wild type) in the *Arabidopsis thaliana* cDNA and R/S ratio in tobacco cDNA was compared. As the result(Table 6), the R/S ration in tobacco cDNA was found to be over ten times higher, comparing with the R/S ratio in the *Arabidopsis thaliana* gene. This difference was highly unexpected, when the high homology of the amino acid sequence between both protoporphyrinogen oxidase was taken into consideration.

Table 6 Resistance to Compound A analogous (*E. coli* liquid culture method with shaking)

Compound	I_{50} (nM) *Arabidopsis thaliana*			Tobacco			Superiority c)
	Wild	Resistant	R/S[a)]	Wild	Resistant	R/S[b)]	
Comp.B	21	18,000	860	2.2	>20,000	9,100	>10.6
Comp.C	3.0	1,800	600	0.25	2,200	8,800	14.7
Comp.D	0.14	280	2,000	0.027	670	25,000	12.5
Comp.E	4.2	4,000	950	0.52	6,300	12,000	12.6
Comp.F	23	6,400	280	3.2	15,000	4,700	16.8
Comp.G	19	8,700	460	3.1	17,000	5,500	12.0

a,b)
Resistant type / Wild type
c)
b/a

Example 8. Resistance to Compound A in transformed plant
A. Construction of a vector for plant transformation

Wild type and resistant type protoporphyrinogen oxidase cDNA of Tobacco and *Arabidopsis thaliana* were inserted into T-DNA in binary vector pBI121 (manufactured by Clontech Co., Ltd.) used for GUS gene introduction. At first, pBI121 was digested with *Sac*I, and made to be blunt end with T4DNA polymerase (manufactured by Takara Shuzo Co., Ltd.). The DNA fragment thus obtained was digested with *Bam*HI, then, after separation to a GUS gene fragment and a plasmid fragment by agarose gel electrophoresis, the plasmid fragment was recovered. On the other hand, pCR-HC having tobacco wild type protoporphyrinogen oxidase cDNA, pCR-RC having tobacco resistant type protoporphyrinogen oxidase cDNA, pBAtPX-C having *Arabidopsis thaliana* wild type protoporphyrinogen oxidase cDNA and pBAtPX-RC having *Arabidopsis thaliana* resistant type protoporphyrinogen oxidase cDNA were digested with *Xho*I, respectively, then, made to be blunt end with a Klenow fragment of DNA polymerase I (manufactured by Takara Shuzo Co., Ltd.). The DNA fragment thus obtained was digested with *Bam*HI, then, after separation to the protoporphyrinogen oxidase gene fragment and a plasmid fragment by the agarose gel electrophoresis, the protoporphyrinogen oxidase gene fragment was recovered.

The plasmid fragment derived from pBI121 and each protoporphyrinogen oxidase cDNA fragment were ligated using DNA Ligation Kit (manufactured by Takara Shuzo Co., Ltd), and

E.coli was transformed and spread on LB agar medium containing kanamycin and incubated at 37°C for overnight.

After the culture, some colonies which showed resistance to Kanamycin were selected, and cultured with shaking in LB liquid medium containing Kanamycin for overnight, then, plasmid DNA was prepared by the alkaline lysis method. The plasmid DNA was digested with restriction enzyme, and *E. coli* having a plasmid in which each protoporphyrinogen oxidase cDNA was inserted was selected. The binary vector in which tobacco wild or resistant type protoporphyrinogen oxidase gene was inserted were named as pBI-NtPX-HC or pBI-NtPX-RC (Figure 4), respectively, and the binary vector in which *Arabidopsis thaliana* wild or resistant type protoporphyrinogen oxidase gene was inserted were named as pBI-AtPX-HC or pBI-AtPX-RC (Figure 5), respectively, and glycerol was added at the final concentration of 15% to the *E. coli* culture and it was stored at -80°C.

B. Introduction of a plant transformation vector into *Agrobacterium tumefaciens*

The four kinds of binary vector above-mentioned were introduced into *Agrobacterium tumefacience* LBA4404 strain (manufactured by Clontech Inc.), using the Triparental mating method. That is, *E.coli* HB101 strain having binary vector, *E.coli* HB101 strain (Clontech Inc.) having helper plasmid pRK2013 and *A. tumefacience* LBA4404 strain were cultured at 28 °C for two days in agar medium for bacterial culture, then the three strains were sufficiently mixed, and cultured at 28 °C for further two days. The mixture was streaked onto AB agar medium having kanamycine and streptomycin (0.3% K_2HPO_4, 0.1% NaH_2PO_4, 0.1% NH_4Cl, 0.03% $MgSO_4 \cdot 7H_2O$, 0.5% glucose, 1.5% agar for bacterial culture). After four days at 28 °C, single colony was picked up and incubated in AB medium containing kanamycin and streptomycin 28 °C for four days, then the plasmid was confirmed by the alkaline lysis method. To the *A. tumefacience* LBA4404 strain was added glycerin to become 15%, stored at -80°C.

C.Production of a transformed plant by *A. tumefacience*

The *A. tumefacience* having the binary vector which had been stored at -80°C was cultured with shaking at 27°C for 24 hours in 523 liquid medium (1% sucrose, 0.8% Bacto-tryptone, 0.4% Bacto-yeast extract, 0.2% K2HPO4, 0.03% MgSO4·7H2O) containing kanamycin. A disk (9 mm diameter) of a tobacco (*N. Tabacum* var. SR1) leaf which had been surface sterilized was immersed for one minute into the culture of the *A.tumefacience*, and then the culture liquid attached thereon was removed with a sterilized paper towel. The leaf disk was transferred into MS agar medium containing 0.6% agar in which

2 ppm NAA and 0.2 ppm BA were added, with the reverse side of leaf being made upside, and incubated at 25°C for 48 hours. The leaf disk was transferred into MS liquid medium containing 500 ppm carbenicillin, 200 ppm claforan, 2 ppm NAA and 0.2 ppm BA and cultured with shaking at 27°C for 48-72 hours to remove *A. tumefacience*. The leaf disk was transferred into MS liquid medium containing 0.6% agar, 100 ppm of carbenycillin, 200 ppm of claforan and 150 ppm of kanamycin, 2 ppm NAA and 0.2 ppm BA and incubated at 27°C for 2-3 weeks and formation of shoot was induced. The shoot was transferred into medium for root development (½ MS, 0.02 ppm IBA, 1.5% sucrose, 0.2% gellan gum) and development of root was introduced. The young plant thus obtained was transferred into culture soil, and was grown in a green house.

D. Confirmation of the introduced gene in the transformed plant

The introduced gene in the transformed plant was confirmed using the PCR method. That is, genomic DNA was extracted from the leaf of the transformed plant, and purified by the CTAB method. Using Taq DNA polymerase, a forward primer for CaMV 35 S promoter (5'-CAC AGA TGG TTA GAG AGG CTT ACG CAG-3', SEQ ID No.9), a reverse primer for NOS terminator (5'-TCA TCG CAA GAC CGG CAA CAG GAT TCA-3', SEQ ID No. 10), and using the obtained genome DNA as a template, and in a thin wall tube, one cycle of 94°C 3 minutes, and 30 cycles of 94°C 20 seconds, 62°C 30 seconds and 72°C 3 minutes were conducted. A part of the product was analyzed by agarose gel electrophoresis, approximately 2.7kb DNA fragment was observed in more than 80% of the plant.

E. Examination for resistance to compound A

From the transformed plant with which the confirmation of introduction of gene was finished, and non-transformed plant used as a control, a leaf almost the same position of the leaf was picked and 9 mm diameter leaf disks were prepared, and floated on a dish in which 0, 125, 250, 1,250, 2,500, 5,000, 12,500nM of compound A aqueous solution were contained. After incubation at 27°C for one week under continuous strong light, the bleaching of the leaf disks was observed, and the permissive concentration to compound A was calculated. As a result (Table 7), in case that the tolerant type of protoporphirinogen oxidase cDNA from tobacco was introduced by pBI-NtPX-RC, more than 100 times of tolerance was observed comparing to the control non-transformed plant. However, in case that rsistant type cDNA from *Arabidopsis thaliana* was introduced by pBI-AtPX-RC, 4 times of resistance was observed comparing to the control, and in case that the wild type cDNA was introduced, high resistance was not observed in each case. Accordingly, it has now been found that in order to express tolerance to photobleaching herbicide, the tobacco tolerant

type protoporphyrinogen oxidase cDNA of the present invention is useful.

Table 7 Resistance to compound A in the transformed tobacco plant level (the Leaf disk method)

Introduced gene (Vector)	Permission conc. to compound A	Resistance
None	125nM	X 1
Arabidopsis thaliana Wild type(pBI-AtPX-HC)	250nM	X 2
Arabidopsis thaliana resistant type(pBI-AtPX-RC)	500nM	X 4
Tobacco Wild type(pBI-AtPX-HC)	500nM	X 4
Tobacco resistant type(pBI-AtPX-RC)	> 12,500nM	> X 100

The present inventors have investigated in the protoporphyrinogen oxidase of a higher plant, and as the result, succeeded in the cloning and expression of the protoporphyrinogen oxidase gene tolerant to photobleaching herbicide, derived from tobacco. When tolerance of the present gene product to photobleaching herbicide was compared to that of protoporphyrinogen oxidase derived from *Arabidopsis thaliana* which has already been reported, it has now been found that the tolerance of the present gene product is far superior, in spite of the high homology in the structure of both gene.

Industrial availability

Protoporphyrinogen oxidase which is new and tolerant to photobleaching herbicide has been obtained, thus, by expressing the enzyme in a host plant, production of a plant which is highly tolerant to light requiring herbicides is possible.